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Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli

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T. HAMOUDA AND J.R. BAKER, JR. 2000. Two surfactant lipid preparations (SLPs) were investigated to determine their mechanism of antimicrobial action. 8N8, a water-in-oil emulsion, and W60C, a liposome, both have bactericidal activity against Gram-positive bacteria and non-enteric Gram-negative bacteria. Additionally, W60C is bactericidal for enteric Gram-negative bacilli when suspended in deionized water. Zeta potential measurements suggested that the resistance of Gram-negative bacilli to 8N8 might be caused by ionic repulsion. Addition of 50 μmol l^{-1} ethylene diamine tetra acetic acid in 100 mmol l^{-1} Tris buffer to either SLPs yielded efficient bactericidal activity against Gram-negative bacilli. This appeared to be due to disruption of the outer membrane and the chelation of divalent cations, as the addition of excess calcium inhibited the antimicrobial effect. Electron microscopy studies documented that 8N8 disrupts the bacterial cell wall, lysing the bacteria, while W60C fuses and internalizes within the cell, causing damage without immediate cell lysis. Understanding the mechanisms of action of these biocidal formulations will help to produce improved formulations with broader spectra of activity.

INTRODUCTION

Most disinfectants have variable biocidal effects against different micro-organisms. Vegetative bacteria are sensitive to a variety of chemical and physical agents, while bacterial spores are more resistant (Borick 1968; Roberts and Hitchins 1969; Hugo and Russell 1982). Antibiotic resistance among bacteria has increased in recent years, and concerns have been raised that cross resistance might develop in bacteria due to exposure to antibiotics or biocides (Rutala 1996; Russell *et al.* 1999). More effective disinfectants can be extremely irritant and toxic, resulting in health complications such as contact dermatitis and mucous membrane irritation among personnel (Hansen 1983; Beauchamp *et al.* 1992). Thus, there is a continuing need for effective and safe biocidal agents for topical and surface disinfection as micro-organisms change and resistant strains develop.

The biocidal activity of surfactant lipid preparations (SLPs) was investigated. These compounds are made of detergents emulsified in oil and suspended in water. They disrupt bacteria through alterations of lipid membranes and are effective within 10–20 min of contact. However, different lipid surfactant formulations have various antimicrobial spectra, suggesting different mechanisms of action. This may relate to the different detergents used in different SLPs, such as cetylpyridinium chloride, Triton X-100 and Tween-60, as well as solvents added to stabilize the emulsion. While these detergents are known to have irritant effects on skin and mucous membranes (Helenius and Simons 1975; Tagesson and Edling 1984), the oils in the SLPs reduce these effects. It is of interest that the detergents and solvents that stabilize the structure of the emulsion also provide the antimicrobial activity of the SLPs.

The biocidal activity of 8N8 and W60C was extensively investigated in this laboratory (Hamouda *et al.* 1998, 1999). These two structures are inherently different; 8N8 is a stabilized emulsion (single lipid layer on the surface of the oil phase) while W60C is a liposome, having a lipid bilayer at the interface of the oil and water phases. The two formulations also have different spectra of antimicrobial activity; 8N8 does not kill enteric Gram-negative organisms, while W60C kills these organisms only when suspended in distilled water. This study was aimed at investigating the possible mechanisms of action of both formulations.

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MATERIALS AND METHODS

Surfactant lipid preparations (SLPs)

8N8 is a water-in-oil nanoemulsion made from 64% soybean oil, 8% tri-η-butyl phosphate, 8% Triton X-100 and 20% water. The stock solution contained 80% lipid components and 20% water (Hamouda *et al.* 1999). W60C is a liposome made of 1% Tween-60, 0·1% halogen-containing cetylpyridinium chloride (CPC), 3% glycerol monooleate, 1% refined soya sterols, 20% soybean oil and 75% water. The stock solution contained 25% lipid components and 75% water (Hamouda *et al.* 1999). The average size of these nanoemulsions is in the range of 400–800 nm. They are stable for at least one year at room temperature (Hamouda *et al.* 1999). The solutions were stored at room temperature and were diluted to the working dilution before every experiment.

Bacterial strains

Escherichia coli (ATCC 11775) was purchased from the American Type Culture Collection (Rockville, MD, USA). *Vibrio cholerae* O1 Eltor biotype, serotype Ogawa was kindly provided by Dr Victor Dirita (Department of Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, USA). *Salmonella typhimurium* is a clinical isolate which was kindly provided by Carol Young (Clinical Pathology Laboratory, University of Michigan, Ann Arbor, MI, USA). The identity of all bacteria was confirmed by the University of Michigan Medical Center Microbiology Laboratory. Bacterial stocks were maintained at −80 °C in Microbank tubes (Prolab diagnostics, Ontario, Canada).

Bacterial media and growth conditions

Escherichia coli and *Salmonella typhimurium*, were grown on Trypticase Soy Agar (TSA). *Vibrio cholerae* was grown on Brain Heart Infusion (BHI) agar. Media were purchased from BBL-Becton Dickinson and all bacterial cultures were incubated at 37 °C.

In vitro bactericidal suspension test

Bacteria were grown overnight on solid medium and then subcultured in broth medium for 3–5 h before testing. Bacteria were centrifuged for 5 min at 1000 *g* using a bench top Sorvall RT6000B (Newtown, CT, USA). The pellet was suspended in 100 μl sterile deionized water (diH_2O) and then added to 25 ml of the dilution fluid. *Escherichia coli* and *Salm. typhimurium* were diluted in either diH_2O or media. *Vibrio cholerae* was diluted in BHI broth only, due to its osmotic susceptibility to water. All bacterial suspensions were adjusted to $1{\cdot}5 \times 10^8$ colony-forming units ml^{-1} (cfu ml^{-1}) based on 0·5 McFarland standard for bactericidal testing (Hendrichson and Krenz 1991). Equal volumes of bacterial suspension and SLPs (at 2× final concentration in diH_2O, tap water or media) were mixed and incubated at 37 °C on a tube rotator for 15 min. Tenfold serial dilutions from 10^{-1} to 10^{-5} were made and duplicate aliquots plated. Plates were incubated at 37 °C overnight and colonies were counted.

To check the effect of ions on the SLPs' bactericidal activity, the bacteria and SLPs were prepared in 50 μmol l^{-1} ethylene diamine tetra acetic acid (EDTA) diluted in 100 mmol l^{-1} Tris buffer (pH 8·0). In some experiments, 10 mmol l^{-1} $CaCl_2$ and/or 5 mmol l^{-1} $MgCl_2$ were added to determine whether they could inhibit the SLPs' bactericidal activity.

Bactericidal activity was calculated as the log reduction in bacterial count following treatment.

Zeta potential measurement

Zeta potentials were determined in order to obtain information on the net charge on the surfaces of the emulsion particles. Nanoemulsions (0·1%) were prepared in diH_2O and 1 mmol l^{-1} NaCl was added to obtain the necessary conductivity for electrophoresis. Zeta potential was measured using a Doppler electrophoresis light scattering analyser (DELSA 440, Coulter, Miami, FL, USA).

Electron microscopy

Vibrio cholerae diluted in media, and *E. coli* diluted in media or water, were treated with 2% of the different SLPs for 15 min at 37 °C. The treated bacteria were washed twice with saline to remove any residual SLPs. After centrifugation, the pellets were fixed with 4% glutaraldehyde in 100 mmol l^{-1} cacodylate (pH 7·3) overnight, then post-fixed with 2% osmium tetroxide in 100 mmol l^{-1} cacodylate for 1 h at room temperature. Finally, the pellets were prepared for routine transmission electron microscopy and stained using uranyl acetate and lead citrate. The bacterial samples were examined with a Philips EM400T transmission electron microscopy (accelerating voltage = 60KV, magnification = 10 000× and magnification factor = 2·5×; FEI Company, Hillsboro, OR, USA).

Statistics/data interpretation

At least four independent experiments were performed; the mean and standard error were calculated when applicable using Microsoft Excel software (Microsoft Corporation).

RESULTS

Zeta potential measurement

8N8 (0·1%) had a mean zeta potential of −13·1 mV, while 0·1% W60C had a mean zeta potential of +31·9 mV. Higher concentrations of SLPs were too turbid to be evaluated.

Bactericidal activity of 8N8 and W60C

W60C (10%) resulted in more than a 6 log reduction in bacterial counts of *E. coli* and *Salm. typhimurium* when diluted in diH_2O. However, W60C had no bactericidal activity against these bacteria in medium. 8N8 did not have any bactericidal activity against enteric Gram-negative bacilli (Fig. 1).

Bactericidal activity of the nanoemulsion in the presence of EDTA/Tris

8N8 or W60C (1%) with 100 mmol l^{-1} Tris buffer prepared in diH_2O showed less than 1 log reduction against the Gram-negative bacilli investigated. The addition of 50 μmol l^{-1} EDTA to these diluted SLPs resulted in more than a 6 log reduction in bacterial count in 15 min. EDTA (50 μmol l^{-1}) without these SLPs did not result in any significant bactericidal activity (Fig. 2). The same treatment, performed in 10% sucrose solution to protect bacteria with damaged cell walls from osmotic lysis, resulted in similar bactericidal activity (data not shown). SLPs/100 mmol l^{-1} Tris/50 μmol l^{-1} EDTA prepared in tap water instead of diH_2O inhibited the bactericidal activity of the emulsion completely (data not shown). Furthermore, the addition of 10 mmol l^{-1} $CaCl_2$ and/or 5 mmol l^{-1} $MgCl_2$ to the treatment mixture abolished its bactericidal effect (data not shown).

Electron microscopy

Electron micrographs of *V. cholerae* treated with 2% 8N8 diluted in media showed disruption of the bacterial cell wall and lysis (1·9 log reduction in bacterial count). The remaining cells appeared to be ghost cells with aggregation of darkly stained granules. *Vibrio cholerae* treated with 2% W60C diluted in media did not show any visible difference compared with the untreated cells, and the cells were viable after treatment (Fig. 3).

Electron micrographs of *E. coli* treated with 2% W60C diluted in water showed condensation and clumping of the cytoplasm with extensive internal vacuolization. This treatment resulted in 1·1 log reduction in bacterial count. However, *E. coli* treated with 2% 8N8 diluted in water did not show a marked variation from the normal control, and cells were viable after treatment (Fig. 4). The osmotic effect due to the treatment of the *E. coli* in water resulted in apparently unhealthy cells (irregular cell walls and increased internal vacuoles) in both the control and 8N8 treatment, even though these cells remained viable after treatment. *Escherichia coli* treated in media looked similar to untreated cells, and they remained viable after treatment (data not shown).

DISCUSSION

Several formulations of surfactant lipid preparations (SLPs) were tested which demonstrated non-toxic, yet effective bactericidal (Hamouda *et al.* 1998), sporicidal (Hamouda *et al.* 1999) and virucidal activity (Donovan *et al.* 2000). In this study, the formulations investigated were 8N8 and W60C, two formulations with different microstructures. These have been shown to be effective against Gram-positive bacteria and spores, as well as some Gram-negative bacilli (e.g. *Vibrio cholerae*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (in preparation)). However, it was interesting to note that there was a difference in the antimicrobial spectrum between the formulations; 10% W60C diluted in water was bactericidal for *E. coli* and *Salm. typhimurium*, but 10% 8N8 had no effect against Gram-negative enteric bacilli whether diluted in water or in media. This suggested differences in the mode of antimicrobial action.

The outer membrane of Gram-negative organisms is unique due to the high content of lipids. The relative resistance of the cell wall of Gram-negative bacteria to dissociation by detergents, disinfectants and antiseptics is well documented (Nixdorff *et al.* 1978; Nikaido 1994; McDonnell & Russell 1999). Nixdorff *et al.* (1978) proposed that the presence of large amounts of lipopolysaccharides (LPS) and proteins, together with very little phospholipid in the outer leaflet of the outer membrane, is the decisive factor in the effective detergent resistance of this membrane. Vaara (1992) explained that the outer membrane of Gram-negative bacteria is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its LPS-covered surface. The outer membrane of these bacteria is also resistant to neutral and anionic detergents. Based on zeta potential measurements, W60C carries a positive charge while 8N8 carries a slightly negative charge. As the bacterial surface of enteric Gram-negative bacilli has a negative charge (Harkes *et al.* 1992), it is postulated that the differential resistance of the enteric Gram-negative bacilli to 8N8 might be caused by ionic repulsion forces resulting from identical charges on the surface of the emulsion particles and bacteria. In contrast, the positive charge on the surface of W60C facilitates its attraction to the negatively-charged bacteria in diH_2O.

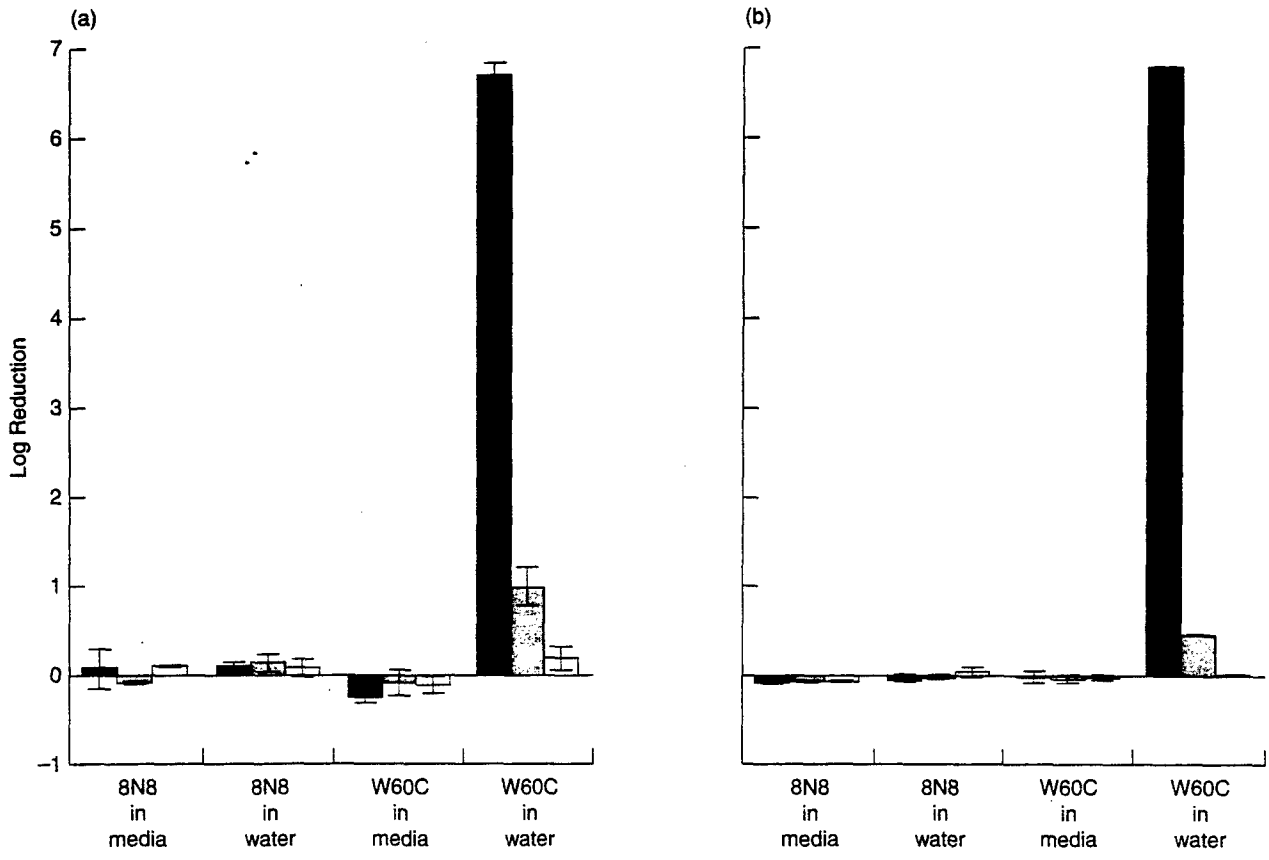

Fig. 1 Log reduction of enteric Gram-negative bacilli treated with various concentrations (■), 10%, (▤), 1% and (□) 0·1% of 8N8 or W60C diluted in water *vs* media for 15 min. More than a 6 log reduction in bactericidal activity was achieved when the bacteria were treated with 10% W60C in water. No killing was observed in any of the other treatments. (a) *Escherichia coli*; (b) *Salmonella typhimurium*

These ionic attraction forces enhance the interaction between W60C and the bacterial cell. Supporting this idea, dilution of W60C in bacteriological media prepared with tap water or in the presence of divalent ions abolished its bactericidal activity, probably due to loss of charge interaction.

Leive (1974) postulated that addition of EDTA to Gram-negative bacilli increases cell permeability by binding divalent cations and releasing some LPS components, leaving an LPS depleted membrane in which the permeability barrier has been partially destroyed. Although the LPS probably rearranges within the membrane when medium is added to the cells, only when new LPS is synthesized can the barrier be completely restored (Leive 1974). Other investigators have used EDTA to increase the permeability of Gram-negative bacteria to several drugs, antimetabolites, detergents, enzymes and enzyme substrates (Ayres *et al.* 1999). The permeability increase is caused by a partial breakdown of a passive permeability barrier due to disruption of the outer membrane. The increase in permeability can be very rapid, from 50 s to 5 min, and it could be terminated by the addition of divalent cations (Leive 1968; Ayres *et al.* 1998). These changes are greater when the cells are suspended in Tris buffer at an alkaline pH. The data suggest that Tris, which in itself can chelate metals, interacts with EDTA to potentiate the binding of divalent ions (Repaske 1958; Neu 1969). EDTA binding of Ca^{2+} and Mg^{2+} on the cell surface, which results in the loss of LPS, is not sufficient to explain permeability changes. Loss of ions may be followed by other changes, such as a loss or alteration of material in the envelope of the organism. LPS-truncated Gram-negative mutants also showed increased sensitivity to antibiotics, which has been attributed to loss of the permeability barrier in this damaged LPS (Leive 1974).

In an attempt to improve the bactericidal activity of W60C and 8N8 against Gram-negative bacteria, these agents were tested in the presence of 50 μmol l^{-1} EDTA and 100 mmol l^{-1} Tris buffer (pH 8). Treatment in EDTA/Tris enhanced the SLPs' bactericidal activity

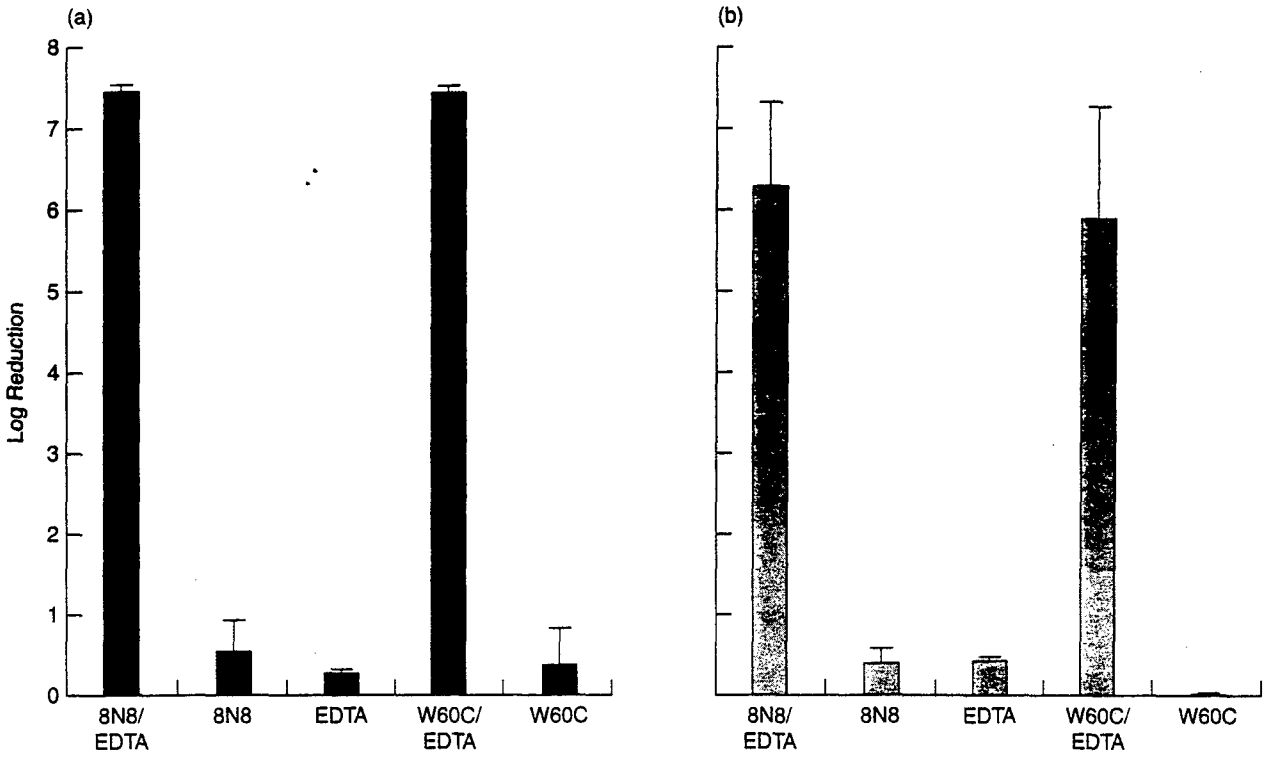

Fig. 2 Log reduction of enteric Gram-negative bacilli following treatment with 1% 8N8 or 1% W60C diluted in 100 mmol l^{-1} Tris buffer and 50 mmol l^{-1} EDTA for 15 min. 8N8 or W60C mixed with EDTA in Tris buffer demonstrated approximately a 6 log reduction in bacterial count. In contrast, 8N8, W60C or 50 mmol l^{-1} EDTA diluted in Tris did not result in any significant bactericidal activity. (a) *Escherichia coli*; (b) *Salmonella typhimurium*

against the enteric Gram-negative bacilli over that observed with diH_2O. This concentration of EDTA was not toxic to the bacterial cells in the assay time (15 min). W60C (1%) in EDTA/Tris (prepared in diH_2O) resulted in more than a 6 log reduction in bacterial count in the Gram-negative bacilli, while no killing was demonstrated from 1% W60C diluted in Tris buffer. Impairment of the permeability barrier with EDTA and Tris buffer allowed 8N8, previously ineffective against these organisms, to demonstrate bactericidal activity similar to W60C. The addition of excess Ca^{2+} and/or Mg^{2+}, or the preparation of the EDTA/Tris buffer in tap water, inhibited the bactericidal activity completely.

From these experiments, it is concluded that the interaction between the SLPs and the bacteria is primarily facilitated by the surface charge on these SLPs. As both 8N8 and enteric Gram-negative bacilli are negatively charged, they repel each other, preventing direct contact. On the other hand, W60C is positively charged, and this allows for fusion with bacterial cells and bactericidal activity. The presence of divalent cations, either from tap water or from media, interferes with this interaction, inhibiting bactericidal activity. The addition of EDTA/Tris to the reaction mixture results in chelation of ions, facilitating the interaction between SLPs and the bacterial cells. EDTA also directly affects the outer membrane, and increasing the permeability of the Gram-negative bacteria to 8N8 results in cell lysis. The addition of divalent ions to the mixture abolishes the SLPs' bactericidal activity due to stabilization of the bacterial outer membrane through interference with the effect of EDTA (Tamaki *et al.* 1971).

In electron micrographs, *V. cholerae* treated with 8N8 in media showed disruption of the bacterial cell wall and lysis. Whatever remaining cells were present appeared as ghosts, with aggregation of darkly stained granules. As treatment of Gram-negative bacilli with 8N8 (diluted in EDTA/Tris prepared in 10% sucrose solution) still resulted in killing, the 8N8 bacterial cell lysis is not due to osmotic changes but appears to result from direct disruption of the cell membrane. In contrast, 8N8 has no bactericidal activity against *E. coli*, and no electron microscopic changes were

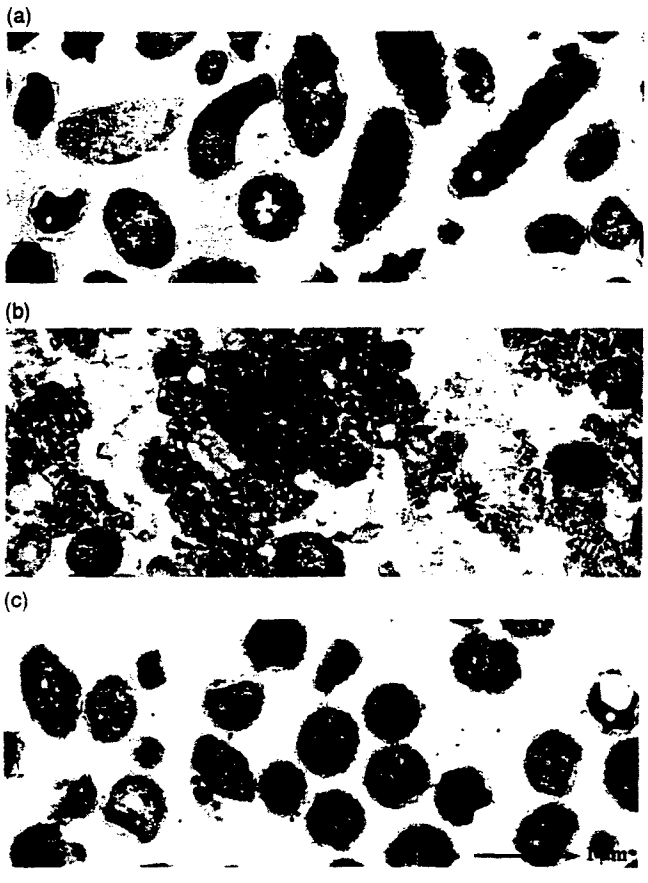

Fig. 3 Electron micrograph of *Vibrio cholerae* reconstituted in BHI. (a) Normal control cells growing in BHI; (b) bacteria treated with 2% 8N8 diluted in BHI for 15 min at 37 °C showing disruption of the cell wall and lysis, with a few ghost cells that have darkly stained granules (there was a 1·9 log reduction in bacterial count); (c) treatment with 2% W60C in BHI did not show any observable difference in cell morphology compared with control cells, and there was no reduction in bacterial count

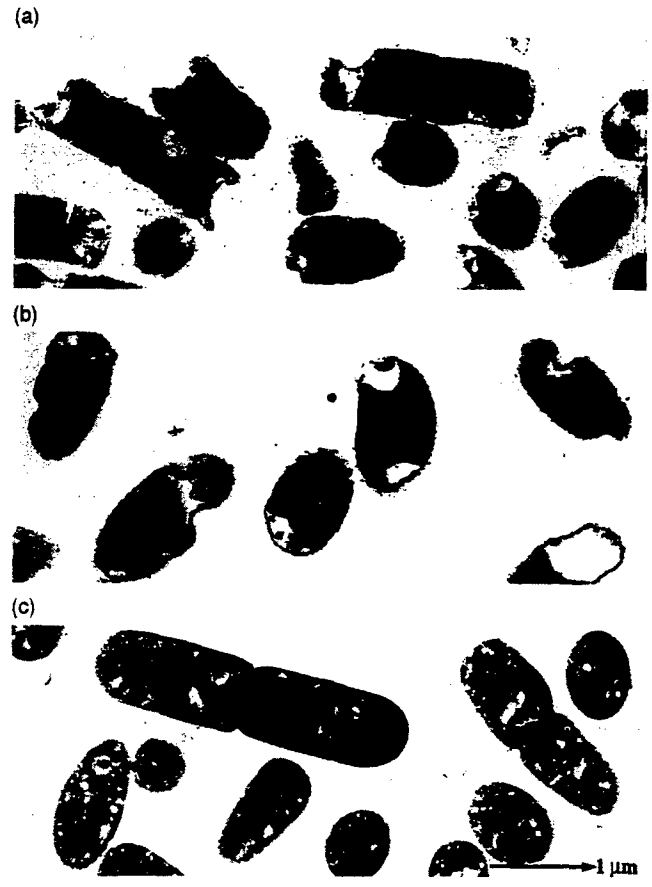

Fig. 4 Electron micrograph of *Escherichia coli* reconstituted in diH_2O. (a) Control cells showing near normal morphology with some irregularities in the cell wall due to their presence in water; these cells were viable. (b) Treatment with 2% 8N8 diluted in diH_2O for 15 min at 37 °C showing near normal cells (no reduction in bacterial count). (c) Treatment with 2% W60C in diH_2O resulted in condensation and clumping of the cytoplasm with extensive internal vacuolization (there was a 1·1 log reduction in bacterial count)

observed in *E. coli* treated with 8N8 either in water or media. *Vibrio cholerae* was not tested in water due to its osmotic fragility.

W60C-treated *Vibrio* are intact in electron micrographs, indicating a mechanism of action different from that of 8N8. Electron micrographs of *E. coli* treated with W60C in water showed intact organisms with extensive cytoplasmic vacuoles; these cells were not viable. In contrast, *E. coli* treated with W60C in media did not show any vacuoles. This could be explained by the hypothesis that W60C, which is a liposome, acts by fusing and internalizing within the cell without immediate cell lysis. The vacuoles resulting from W60C treatment may be the result of water and lipid uptake with the liposome. When W60C treatment was performed in media, no vacuoles were detected, suggesting that fusion and uptake did not occur. The bacterial cells were also viable, again suggesting that no interaction between W60C and the bacterial cells had occurred.

These SLPs have a potent biocidal activity with minimal toxicity, as well as being non-irritant and non-corrosive (Hamouda *et al.* 1999). As a result, they could prove useful in many applications as a broad spectrum decontamination agent for the environment and people. Understanding the mechanism of the bactericidal activity of the SLPs is of crucial importance for the development and enhancement of these formulations. Currently, several new SLPs developed from the findings observed in these experiments are

being investigated. These formulations appear to have broader biocidal activity and may find utility in a wide variety of disinfectant applications.

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